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(54) Title: DUPLEX-FORMING, POLYNUCLEOTIDE CONJUGATES

(57) Abstract

The invention provides ligand-binding duplexed structures having significantly enhanced stability under physiological conditions. The structures are provided in the form of polynucleotide conjugates capable of adopting a duplexed structure, in which annealable polynucleotide strands are coupled covalently at one or both ends through a chemical linker which establishes a stabilizing bridge between strands. Various applications for the stabilized duplexed structures are described, including therapeutic applications for example in the treatment of AIDS.

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DUPLEX-FORMING, POLYNUCLEOTIDE CONJUGATES

This invention is in the field of nucleic acid chemistry. More particularly, the invention relates to polynucleotide conjugates that adopt a ligand binding duplexed structure, to the production of such conjugates particularly via automated synthesis techniques, and to their use in therapeutic, diagnostic and other applications.

Background to the Invention

The ability to regulate cellular processes at the genetic level in a highly selective and therapeutic manner is now offered by various forms of oligonucleotide-based pharmaceuticals. These oligonucleotides are designed according to their nucleic acid sequence to arrest genetic processes by binding disruptively to a selected genetic target, usually a viral gene or a human gene that is associated with a particular disease state such as cancer or a condition such as inflammation. Transcription of an undesired gene can, for example, be arrested by a synthetic oligonucleotide that hybridizes selectively to a control region or coding region of that gene; similarly, translation of an undesired protein can be arrested using an oligonucleotide that hybridizes with a control region or coding region of the messenger RNA encoding that protein. Many of the problems associated with the practical use of such oligonucleotide-based therapeutics, such as cell uptake, stability, and cost of production, have been resolved by recent advances in nucleic acid chemistry.

These current strategies contemplate principally the use of oligonucleotides which, in order to hybridize to their intended nucleic acid target, are necessarily single-stranded complements of that target. That is, oligonucleotides intended for use as pharmaceuticals are designed currently to bind as single-stranded entities to other nucleic acid targets, whether single-stranded messenger RNA or single stranded DNA (the so-called "sense" and "anti-sense" approaches, reviewed for example by

Uhlmann et al, 1990, Chemical Rev., 90:543) or, as has more recently been proposed, to double stranded DNA (the "triplex" approach). These approaches neglect other cellular targets that are at least equally attractive in the overall development of gene regulating therapeutics. More particularly, it would be desirable to provide oligonucleotide agents capable of interfering with interactions specifically between nucleic acids and their ligands, particularly their protein ligands, having a role in infectious and other disease states.

The feasibility of designing oligonucleotides that interfere with a protein/nucleic acid interaction of therapeutic interest is complicated in that, in the majority of instances, the protein recognizes a nucleic acid that is double stranded in structure; and further in that double stranded oligonucleotides of the small size necessary for pharmaceutical applications, for uptake by the cell, and for stability, are highly unstable and must typically be incubated under temperatures so cold and/or salt concentrations so high as to make subsequent study and use of the duplexed structures impractical.

It is a principle object of the present invention to provide polynucleotide conjugates that are capable of adopting a ligand-binding duplexed structure.

It is a further object of the present invention to provide ligand-binding polynucleotide conjugates of enhanced stability, i.e. of enhanced physical or chemical stability.

It is another object of the present invention to provide a process for preparing such duplexed structures of enhanced stability.

It is another object of the present invention to provide a compositions for therapeutic use that incorporate, as active ingredient, a

polynucleotide conjugate that is capable of adopting a stability-enhanced duplexed structure that binds with a target ligand of therapeutic interest.

Summary of the Invention

There is provided by the present invention a family of duplex-forming compounds, herein referred to as polynucleotide conjugates, which comprise a first polynucleotide strand having an end, a second polynucleotide strand having an end and which is capable of annealing with the first polynucleotide strand to form a ligand binding structure, and a chemical linker which is coupled between ends of the strands to form a bridge permitting the conjugate to form a ligand-binding duplexed structure.

According to one aspect of the present invention, the stability-enhanced duplexed structures of the invention are provided in the form of linear polynucleotide conjugates, conforming to the general formula:

X - L - Y (I)

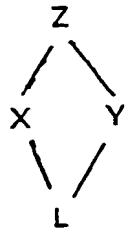
wherein:

X is a polynucleotide having a 3'terminus;

Y is a polynucleotide capable of annealing with X, and having a 5'terminus; and L is a chemical linker coupled between the 3'terminus of X and the 5'terminus of Y to form a bridge permitting the conjugate to form a ligand-binding duplexed structure.

Compounds conforming to the general formula (I) are linear polynucleotide conjugates and are most conveniently produced using automated polynucleotide synthesis techniques. For this purpose, the present invention further provides analogues of the chemical linkers in bifunctional form for incorporation between nucleotide strands using established nucleotide coupling protocols.

The stability-enhanced duplexed structures may also be in the form of cyclic polynucleotide conjugates, which conform either to the general formula:



(IIa)

wherein:

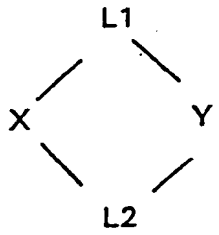
X is a polynucleotide having a 5'terminus and a 3'terminus;

Y is a polynucleotide capable of annealing with X and having a 3'terminus and a 5'terminus;

Z is a polynucleotide coupled covalently between the 5'terminus of X and the 3'terminus of Y; and

L is a chemical linker coupled between the 3'terminus of X and the 5'terminus of Y, to form a bridge permitting the conjugate to form a ligand-binding duplexed structure;

or to the general formula:



(IIb)

wherein:

X and Y are as defined above; and

L1 and L2 are independently selected chemical linkers coupled, respectively, between the 3'terminus of X and the 5'terminus of Y and

the 5'terminus of X and the 3'terminus of Y, to form chemical bridges permitting the conjugate to form a ligand-binding duplexed structure.

The cyclic polynucleotide conjugates of the invention, as represented by formulae IIa and IIb are suitably prepared by synthesizing the linear analogue thereof using the automated nucleotide coupling techniques appropriate for linear conjugates of formula (I) and then closing the linear conjugate typically using either chemical or enzymatic means, to form the cyclic polynucleotide conjugate.

In accordance with another aspect of the present invention, there is provided a pharmaceutical composition which comprises a ligand-binding polynucleotide conjugate of the present invention and a pharmaceutically acceptable carrier. In valuable embodiments of the invention, the polynucleotide conjugate is one capable of adopting a duplexed structure that is recognized by i.e. binds with, a target ligand that is a protein, for example a protein capable of regulating gene expression. In a specific embodiment of the present invention, the polynucleotide conjugate is characterized by an affinity for binding with protein which regulates viral gene expression e.g. the HIV tat protein. Alternatively, the polynucleotide conjugate can be designed by appropriate selection of its component polynucleotide strands and linker(s) to bind with proteins that regulate oncogene expression, or expression of genes implicated in other disease states or medical conditions.

According to another aspect of the present invention, the chemical linker component of the polynucleotide conjugate incorporates a functional group which serves as a site of attachment for a reporter molecule, such as a radiolabel or other diagnostically useful label. Accordingly, the invention further provides detectably labelled analogues of the polynucleotide conjugates, for diagnostic use or for use in assays

designed to measure binding between the duplexed form of the conjugate and a ligand, such as a DNA- or RNA-binding protein. Further, the attachment site within the chemical linker may be exploited to couple the polynucleotide conjugate to an affinity column matrix, for use in extracting ligands from biological sources.

These and other aspects of the present invention are now described in greater detail with reference to the accompanying drawings, in which:

Brief Reference to the Drawings:

Figures 1 and 2 illustrate duplexed structures of various conformations and configurations that can be stabilized in accordance with the present invention. Solid lines illustrate polynucleotide structure and hatching identifies the nucleotide components. The symbol "." is used to indicate hydrogen-bonded base-pairing within annealed regions of the polynucleotide strands, and the symbol L is used to indicate location of the chemical linker;

Figure 3 shows incorporation of a specific linker of the present invention between polynucleotide strands;

Figures 4-7 illustrate the structure of specific polynucleotide conjugates of the present invention; and

Figures 8 and 9 illustrate graphically the cellular uptake of specific polynucleotide conjugates of the invention.

Detailed Description of the Invention

The present invention provides polynucleotide conjugates characterized by the properties of ligand binding and enhanced stability.

In the present specification, the term "enhanced stability" refers unless otherwise stated to the superior thermal stability of a polynucleotide conjugate relative to its unlinked counterpart, as measured using melting temperature (Tm) assays established in the art. The term "ligand"is used herein with reference to agents that bind measurably, in the context of an assay appropriate for that measurment, to nucleic acid structures, principally double stranded structures but also single stranded structures. The term ligand is thus intended to embrace such agents as proteins, including proteins that regulate genetic processes such as transcription and translation, as well as non-protein entities including but not limited to intercalating agents and nucleic acid binding antibiotics as well as other nucleic acids. The term "ligand-binding" is thus used with reference to polynucleotide conjugates that adopt a structure that is bound measurably by a ligand to which the conjugate is targetted.

In providing duplexed structures of enhanced stability, the present invention permits the use of double stranded polynucleotide structures in a wide variety of applications not previously possible, because of prior stability problems. Because the chemically linked duplexed structures of the present invention are substantially more stable than their unlinked counterparts under physiological conditions, for example, therapeutic applications for duplexed structures are now feasible. In addition, it will be appreciated that the stability-enhancing effect of the chemical linker can be exploited to eliminate polynucleotide regions that are otherwise required to permit formation and maintenance of the desired duplexed structure *in vitro* and *in vivo*. Thus, duplexed structures that are much smaller in molecular weight and accordingly more acceptable for therapeutic use, can be produced. Furthermore, the chemical linkers exploited in the present invention are substantially resistant to nuclease digestion, and thus further contribute to duplex stability.

To stabilize polynucleotides, the present invention applies the strategy of incorporating a chemical linker between one or both ends of polynucleotide strands capable of forming a duplexed structure. It will be understood that in order to form a duplexed structure, such strands will share at least a region of sequence complementary sufficient to permit annealing of the strands. The individual polynucleotide strands forming the duplex may consist of RNA or DNA monophosphates or synthetic analogues thereof, or mixtures thereof. Synthetic analogues include for example those incorporating variations in the base constituent, such as thio- and aza-substituted bases; in the sugar consitituent such as alkyl- and halo-substituted riboses and arabinose equivalents; and analogues incorporating variation in the monophosphate group, such as phosphorothioates and dithioates, methyl phosphate and methyl phosphonates, phosphoramidates and phosphoramidites and the like. As is herein described, a polynucleotide strand may also incorporate a non-nucleic acid component, to the extent that duplex formation and ligand binding are not substantially impaired.

The polynucleotide strands forming the duplex may be of the same or different lengths, and each may incorporate any number of nucleotides in the range from 2 to a maximum that is dictated largely by the limits of automated gene synthesis techniques. Strands consisting of not more than about 200 nucleotides, for example not more than about 100 nucleotides, will derive the most benefit from the stabilizing effect of the chemical bridge, however. Preferably each of the polynucleotide strands consists of from 3 to 100 nucleotides, and more preferably, from about 4 to 50 nucleotides. Polynucleotide strands that are capable of annealing, and which can thus benefit from the linker strategy herein described, include those strands that anneal in their anti-parallel orientation i.e. consist of beta nucleotides, and strands that consist of alpha nucleotides in one strand and beta nucleotides in the other strand, and thus can anneal in the parallel orientation. In the simplest case, the polynucleotide

strands will be precisely complementary and equivalent in length, and will anneal along their entire length, to form a completely double stranded duplexed structure. It will be appreciated however, that with the aid of a chemical linker, duplexed structures having a variety of conformations and configurations can be stabilized, in accordance with the present invention. Some of the duplexed structures currently contemplated are illustrated schematically in Figures 1 and 2, to which reference is now made. Other structures or combinations may also be stabilized in accordance with the present invention, of course.

As shown schematically in Figure 1, duplexed structures that can be generated as linear polynucleotide conjugates of the general formula (I) comprise a single chemical linker incorporated at one end of the duplex structure. Figure 1(a) illustrates the simplest case which, as described above, incorporates a linker at one end of precisely complementary polynucleotide strands, which anneal along their entire length to form a fully double stranded duplex structure. Figure 1(b) illustrates the case in which the annealable strands incorporate a terminal mismatch, which results in a non-annealing "fork" structure at one end of the duplex. Figure 1(c) illustrates the situation in which one polynucleotide strand incorporates an internal, mismatched region resulting in a non-annealed bulge. Figure 1(c) further illustrates that polynucleotide strands of different length can also be linked, according to the present invention, as is further shown by the structure of Figure 1(d).

Similarly, duplexed structures that can be generated as cyclic polynucleotide conjugates of the formula (IIa) and (IIb) may also adopt various conformations and configurations. As shown in Figure 2(a), the simplest case is again the situation where precisely complementary strands are coupled using chemical linkers at both ends. Similarly, the forked structure shown in Figure 2(b) can also be linked at both ends, as may the bulged structure shown in Figure 2(c). The forked structure of

Figure 2(b) also illustrates that chemical linkers of different length may be used to bridge polynucleotide strands in the annealing relationship desired for duplex formation. Duplexes that are more elaborate in structure can also be stabilized if desired, as shown for example in Figures 2(d) and 2(e). The duplexed structures appearing in Figures 2(a) - (e) are intended to be embraced by the general formula II(b) recited hereinabove.

The duplexed structure illustrated in Figure 2(f) represents a special but important case, in which a cyclic duplexed structure is created by incorporation of a single chemical linker, as embraced generally by the formula II(a) recited hereinabove. In this case, Z is represented by the polynucleotide 'loop' bridging the annealed polynucleotide strands. As will be described herein, such structures exist naturally in the unlinked form, occurring predominantly in the form of RNA "hairpins" that regulate the expression of certain viral and other genes through a protein-binding interaction. Such duplexed structures are accordingly ideal as targets for therapeutic application, when in their chemically linked form.

As noted above, the linking of duplex-forming polynucleotide strands is achieved by covalently coupling the chemical linker between neighbouring termini of the polynucleotide strands, either between the 5'terminus of one strand and the 3'terminus of the other, or *vice versa*. As is shown in Figure 3, linkers are most suitably incorporated by coupling between the monophosphate or analogous groups borne at the termini. It is to be understood that the chemical linkers used in the present invention are synthetic chemical linkers as opposed to polynucleotide-based linkers of the type represented by substituent Z in Formula (IIa).

The chemical linker has a length selected ideally to preserve the desired annealing relationship between strands at the location of the linker. Since numerous duplex conformations can be stabilized using the

linker, linkers of similarly various lengths can be incorporated for this purpose. Generally, the length of the linker will correspond to the length of a linear chain alkane comprising from about three carbon atoms (C_3) to about 30 carbon atoms (C_{30}). It has been found in particular that a chemical linker having a length equivalent to a linear chain alkane consisting of from 7 to 20 carbon atoms, suitably 8 to 15 carbon atoms and desirably 9-12 carbon atoms, is appropriate to link polynucleotide strands at an annealed location. For coupling of strands at a mismatched, non-annealed location, a chemical linker having a length equivalent to greater than about 10 carbon atoms, for example having a length in the range from about 10 carbon atoms to about 20 carbon atoms, is suitable for incorporation. Since functional groups are also incorporated at the ends of the linker to permit coupling with nucleotides, as described below, determination of desired linker length should be made with this in mind.

The chemical composition of the linker can vary widely, provided that consideration is given to the need for stability under physiological conditions and under the conditions encountered during nucleotide coupling protocols. The linker may contain functional groups, for example to serve as attachment sites for other molecular entities, provided that suitable protecting groups are employed during synthesis of the polynucleotide conjugate. A key requirement in choosing a linker composition is to retain the length appropriate for duplex formation. In this connection, it will be appreciated that side chains are acceptable, particularly in the central region of the linker. Moreover, the desired length of the linker can be achieved using carbon atoms or carbon atoms in combination with heteroatoms, including oxygen, sulfur, phosphorus, nitrogen, etc. Also, cyclic structures can be incorporated, including benzene and heterocycles such as piperidine, piperazine or pyridine coupled within the linker chain either through a carbon center or a heteroatom. It will also be appreciated that the chemical composition of

the linker can be manipulated through component selection to alter hydrophobicity or hydrophilicity, if desired, particularly for the purpose of altering solubility, cellular uptake, and to facilitate dosage formulation where therapeutic applications are being considered.

For incorporation between nucleotides, the chemical linkers are provided in the form of bifunctional analogues, bearing terminal functional groups that, desirably, are amenable to protection and derivatization that adapts them for coupling using the same protocols applied conventionally for automated nucleotide coupling. Such bifunctional linker analogues conform to the general formula,

R - linker - R'

wherein, most suitably, R and R' are independently selected from among the group consisting of -OH, -SH, -NH and functional equivalents of these groups. So that the linkers can be incorporated, and the polynucleotide conjugates synthesized, using the currently most practical phosphoramidite approach, the linker is preferably one in which at least one of R and R' is OH. Most preferably, both R and R' are OH.

Bifunctional linkers suitable for use in coupling polynucleotide strands at an annealed location are exemplified by, and include:

$HO-(CH_2)_n-OH$, n= 6-18

$HO-(CH_2CH_2-O)_n-(CH_2)_2-OH$, n= 2-10

$HO-(CH_2)_mCH=CH-(CH_2)_n-OH$, m,n= 2-10

$HO-(CH_2)_m$-Phenyl-$(CH_2)_n-OH$, m,n= 2-10

$HO-(CH_2)_m$-Phenyl-Phenyl-$(CH_2)_m-OH$, m,n=2-10

$HO-(CH_2)_m-C\equiv C-(CH_2)_n-OH$, m,n= 2-10

$HO-(CH_2)_m$-piperazinyl-$(CH_2)_n-OH$, m,n=2-10

$HO-(CH_2)_m-O-P-(O)_2-(CH_2)_n-OH$, m,n = 2-10

It should be appreciated that linkers of appropriate length may also be formed *in situ* i.e. during conjugate synthesis, by coupling selected linkers sequentially to extend linker length as desired.

The polynucleotide conjugates of general formula (I), which are linear molecules capable of forming duplexed structures can be synthesized by applying now conventional techniques of polynucleotide synthesis, particularly in combination with the commercially available polynucleotide synthesizing devices, or "gene machines". Various strategies of solution and solid phase synthesis can be used, of course, including the phosphotriester method, the solid phase H-phosphonate method or the solid phase phosphoramidite method. The latter is currently the method of choice, for synthesis of polynucleotide-based compounds of the invention. In the phosphoramidite approach, nucleotides that are fully protected are coupled sequentially, in the 3' --> 5' direction, to a first nucleotide that is coupled releasably to a solid support, such as aminopropyl controlled pore glass or polystyrene resin. Nucleotide protecting groups include, for nucleophilic amino functions on the bases, either isobutyryl (N-2 of guanine) or benzoyl (N-6 of adenine and N-4 of cytidine) that are removable upon completion of synthesis by ammoniolysis. In the case of deoxyribonucleotides, the 5' primary hydroxyl of the deoxyribose sugar is protected with an ether moiety, either dimethoxytrityl (DMT) or monomethoxytrityl (MMT), which is removed by mild protic acids at the beginning of each coupling cycle. The 3' secondary hydroxyl function of the deoxyribose sugar is derivatized with the highly reactive phosphoramidite group, either methyl phosphoramidite or ß-cyanoethyl phosphoramidite, which is activated for coupling by a weak acid.

For incorporation into such an automated synthesis procedure, the bifunctional linker analogues of the present invention can be similarly protected and deprotected for coupling. Thus, in the case where the linker analogue bears terminal hydroxyl groups, these may be protected in the same manner as the 5' and 3' hydroxyls of the nucleotides selected for coupling. In other words, one hydroxyl is protected with the ether moiety, such as DMT, and the other is derivatized to provide the phosphoramidite group, to yield a compound of the general structure, DMT-O-linker-O-phosphoramidite. This permits unidirectional incorporation of the linker into the linear polynucleotide, at a desired position along its length.

Techniques for obtaining linkers suitably adapted for nucleotide coupling reaction are provided in Example 1 herein. Briefly, for dimethoxy or monomethoxy tritylation, the trityl halide and a slight molar excess of the diol are reacted in pyridine at room temperature, and the product is recovered after mixing with methanol, resuspension in chloroform and then washing and drying, with solvent removal. The tritylated product can then be phosphitylated, to protect the remaining hydroxyl group, by reaction with 2-cyanoethyl-N,N-diisopropylchorophosphoramidite in the conventional manner. The so-protected diol linker can then be incorporated into an automated nucleotide synthesis protocol in the same manner as would any protected nucleotide.

Thus, to produce a linear polynucleotide conjugate, the resin-bound first nucleotide is treated with protic acid to remove the trityl protecting group at the 5'hydroxyl, the 3'hydroxyl phosphoramidite group of the next nucleotide is activated to allow 3' to 5' coupling, and then oxidized to complete coupling. At the desired point in the sequence, the protected linker is incorporated using the same deprotection/activation strategy and the coupling continues until the linear form of the double stranded oligonucleotide is produced. This is then released from the solid support

and treated to deprotect bases, isolated and then purified using well established protocols.

Figure 3 provides the chemical structure resulting from the covalent coupling of a specific triethylene glycol-derived linker, between polynucleotides. It will be noted that the linker is coupled to the termini of the nucleotides through the monophosphates borne on the respective 5' and 3' hydroxyl groups.

Substantially the same synthesis protocol can be employed for synthesis of RNA-based, linear polynucleotide conjugates, but with use of a blocking group for the 2'hydroxyl, such as the tert-butyldimethylsilyl group (TBDMS) or the triisopropylsilyl group (TIPS), and optionally with use of the MMT or DMT ethers for 5'hydroxyl protection.

For the production of cyclic polynucleotide conjugates of the present invention i.e. those of formulae (IIa) and (IIb), a linear analogue of the cyclic molecule is first produced using the procedure described above for linear polynucleotide conjugate production. The linear analogue is produced such that the ends of the resulting linear conjugate can be closed either by chemical reaction or by enzymatic ligation. Chemical closure can be achieved using various available techniques. One convenient approach requires fully-deprotected linear precursor sequences and use of chemical condensation reagents, such as cyanogen bromide as described by Prakash, G. et al (1992) J. Am. Chem. Soc., 114, 3523-3527, water-soluble carbodiimide as described by Ashley, G.W. et al, (1991) Biochemistry, 30, 2927-2933, and N-cyanoimidazole as described by Luebke, K.J. et al, (1992) Nucleic Acids Res., 20, 3005-3009. An alternative approach requires a fully-protected linear precursor with only a free 5'-OH and a 3'-phosphate selectively deprotected for cyclization (see Rao, M.V. et al, (1989) Nucleic Acids Res., 17, 8221-8239. These linear precursors can be prepared in solution via the phosphotriester approach.

The typical condensation reagent in this case is 1-(2-Mesitylenesulfonyl)-3-Nitro-1,2,4-Triazole (MSNT). After the post-synthesis coupling, the cyclic oligonucleotides are treated according to standard procedures of deprotection and purification. Another alternative approach generates a fully-protected cyclic oligonucleotide directly on the polymer-support (see Barbato, S. *et al.*, (1989) Tetrahedron, 45:4523; and Capobianco, M.L. *et al.*, (1990) Nucleic Acids Res.,18:2661). This phosphotriester approach does not require a post-synthesis cyclization, and results cyclic molecules with high efficiency.

Cyclization of the polynucleotide conjugate can also be achieved by enzymatic ligation of the free ends of a linear conjugate. The ends to be ligated correspond preferably to an annealing site in the duplexed structure, to facilitate action of the enzyme, preferably an RNA or DNA ligase, as appropriate. To anneal DNA ends, the linear conjugate is preferably incubated first under annealing conditions and then treated with either RNA or DNA ligase. RNA ends can be annealed in similar fashion, by treatment with RNA ligase in particular. The cyclic polynucleotide conjugates resulting from the reaction can be recovered and purified using techniques established generally for polynucleotides, and as described in the examples herein.

To provide duplexed structures that, in accordance with the present invention, exhibit not only enhanced stability but also a ligand binding property, the polynucleotide strands to be linked during synthesis are selected in terms of their nucleic acid sequence, and based on knowledge of the particular nucleic acid sequence to which a target ligand binds. It will be appreciated that selection of strands appropriate for desired ligand binding can be guided by the vast scientific literature dealing with protein/nucleic acid interactions. In those instances where a binding domain of specific interest remains to be identified, it will be appreciated that the mapping of that domain can be achieved using

conventional approaches, so that a specific binding sequence can be elucidated. The strategy hereindescribed can in fact facilitate such mapping, by permitting the synthesis of a series of stabilized duplexed structures representing putative ligand binding domains that can then be screened for ligand binding activity using for example the mobility shift assays of the type hereindescribed.

In a preferred aspect of the present invention, the polynucleotide conjugates are employed as mimics of naturally occurring duplexed structures, and the polynucleotide strands in the conjugate are accordingly selected to correspond in sequence to a naturally occurring duplex counterpart. Conceivably, any duplexed region of a naturally occurring gene or other genetic element can be duplicated in stability-enhanced form, in accordance with the present invention.

Ligands of potential interest include those proteins which on binding to their natural, nucleic acid target, directly or indirectly, influence the utilization or fate of that nucleic acid target. Examples of such proteins include: ribo- and deoxyribonucleoprotein complexes; gene regulatory proteins such as repressors, activators and transactivators, etc.; proteins involved in the modifications and fate of mRNA molecules, including splicing, polyadenylation, capping, nuclear export, translation, degradation, etc.; proteins involved in the assembly and utilization of other RNA or ribonucleoprotein structures such as ribozymes, tRNA synthetases, splicing complexes, etc. In all cases, the essential feature of such proteins is that they recognise particular nucleic acid structures on the basis of their conformation and/or sequences; embodiments of this invention would provide effective analogues when they maintain some or all of such requirements.

Also of interest are polynucleotide conjugates that bind ligands other than protein ligands, e.g. chemical ligands such as intercalating

agents (e.g. psoralen and ethidium bromide), nucleic acid-binding antibiotics (e.g. distamycin and netropsin) and other nucleic acid structures.

In a particularly preferred embodiment of the present invention, the polynucleotide conjugates comprise polynucleotide strands which, in their duplexed form, exhibit binding affinity for the tat protein of the human immunodeficiency virus, HIV. Through interaction with the RNA hairpin structure known as Tar, the tat protein mediates a rapid increase in the production of the viral components required for HIV replication, which in turn leads to the onset of AIDS. It has been suggested that agents capable of interfering with the tat/Tar interaction will be useful in arresting HIV replication, and thus efficacious in the treatment of AIDS. The present invention accordingly provides a polynucleotide conjugate which is capable of adopting a duplexed structure having a binding affinity for tat. Such binding affinity is revealed using standard mobility shift assays, in tat/Tar complexes, and thus tat-binding, is revealed by altered migration relative to tat and Tar alone (see Roy et al, *infra*). According to a specific embodiment of the invention, the polynucleotide conjugate has a chemical structure described in the examples herein. It will be appreciated, however, that sequence variation can be tolerated without loss of tat binding affinity, and such variations which retain tat binding are within the scope of this embodiment of the present invention.

Other viral processes can also be targetted for therapeutic interference using the stabilized duplex structures of the present invention. For example, in HIV, besides the TAR structure, the duplexed RRE RNA structure required to regulate splicing and the duplexed $tRNA_{Lys3}$ structure used as a primer for reverse transcription can be mimicked using the present strategy. There may also be produced duplexed structures which bind other regulatory protein ligands, for example those known to exist in human pathogenic viruses, including: the P protein of Hepatitis B

virus (HBV); the vp16 protein of HSV; the E1 and E7 proteins of Papilloma virus (HPV); the BZLF1 and EBNA-1 proteins of Epstein Barr virus (EBV); as well as additional proteins in these and other viruses.

Many other protein ligands and their corresponding nucleic acid targets are known in microbial, plant and animal species. Of particular note are regulatory proteins known such as that which interacts with the heat shock element (HSE), those which mediate inflammatory processes such as the interleukins, and those known to be involved in transforming processes giving rise to cancer, such as the *jun* and *fos* oncogene families, which bind preferentially to particular DNA targets, and such targets can also be reproduced in stabilized duplexed form in accordance with the present invention.

Formulation and administration of the compounds herein described, and indeed any annealed polynucleotide structures having pharmaceutical utility, can be accomplished in accordance with procedures routinely applied to aqueous-soluble compounds. Thus, for parenteral administration, buffered saline solutions are acceptable. Where a reduction in administration frequency is desirable, timed-release polymeric compositions which do not unfavourably chemically modify the compounds are acceptable. Modification of pharmacokinetic properties, especially distribution, are achieved, for instance, through the use of liposomal or cationic lipid formulations.

In an alternative embodiment of the present invention, the polynucleotide conjugates comprise polynucleotide strands which in their duplexed form present nucleic acid epitopes of interest, for example as immunogens suitable for raising antibodies. The raising of such antibodies can be achieved in the manner conventional for polyclonal antibody production, or for monoclonal antibody production. Such antibodies will find utility in assays designed to detect eptiopes against

which the antibodies were raised, especially when conjugated to a suitable reporter molecule; and may also be useful in protecting a region of a polynucleotide while manipulating that polynucleotide at another site.

The polynucleotide conjugate may be coupled via an attachment site incorporated within the chemical linker, to a desired agent such as a cross-linking agent, reporter molecule, cell uptake enhancer such as lipid or cholesterol, alkylating groups, chromatographic beads and other functional groups . A variety of chemical groups may serve as attachment sites, provided of course that such groups permit coupling of the polynucleotide strands as desired, and can be protected during polynucleotide synthesis. Ideally, the attachment site is constituted by a chemical entity that can be protected by a base-labile protecting group removable by ammoniolysis. One such group is the FMOC group used in conventional peptide synthesis protocols. In another embodiment, the attachment site may be constituted by a phosphate group incorporated within the linker, which can be derivatized following oxidation from either H-phosphonate to phosphate triester, or phosphite triester to phosphate triester.

When coupled with a reporter, such as a radiolabel, the conjugates of the invention can may also be used diagnostically e.g. as a competing ligand, to assay specimens for the presence of target ligand in a qualitative or semi-quantitative fashion, for example using a competitive binding assay format.

Example 1 - Dimethoxytritylation of linkers

As a first step in adapting linkers bearing terminal diol groups for incorporation via automated polynulceotide synthesis, one of the two diol groups was first protected using the dimethoxytrityl group. The procedure is generally applicable for any diol linker, and proceeds according to the reaction scheme provided below:

DMTr-Cl + HO-(linker)-OH ----> DMTr-O-(linker)-OH

(i) 10-30mmol of the diol compound was co-evaporated with anhydrous pyridine (3 x 20 ml). The residue was then dissolved in fresh dry pyridine (50-150 ml) to yield a final diol concentration of about 1 mmol/5 ml).

(ii) 4,4'-dimethoxytrityl chloride (6.7-20 mmol) was then added in small portions. The ratio between DMTr-Cl and diol was 1:1.5 eq.

(iii) The reaction was followed at room temperature by thin layer chromatography (TLC) (MeOH/$CHCl_3$, 1:9, v/v) until the appearance of a product spot that was intense relative to remaining DMTr-Cl. The reaction was usually complete after 2-4 hours. The DMTr derivatives were visualized as red-orange spots using an acidic spray (60% aqueous perchloric acid/ethanol, 3:2, v/v).

(iv) When the reaction was complete, 20-30 ml of MeOH was added to quench excess DMTr and the mixture was stirred for an additonal 15 minutes.

(v) The solution was then concentrated to a syrup and the residue was resuspended into 50-150 ml of $CHCl_3$. The chloroform phase was then washed once with 5% $NaHCO_3$ (25-75 ml), and twice with saturated NaCl solution. The aqueous phase was back-extracted with $CHCl_3$ (25-75 ml). The organic phases were combined and dried over anhydrous sodium sulphate. After filtration, the solution was evaporated down to an oily residue under reduced pressure.

(vi) The residue was purified by flash chromatography on silica gel. The column was first eluted with petroleum ether/EtoAc (5:1, v/v) and then with petroleum ether / EtoAc (2:1, v/v).

(vii) Fractions containing the final product were combined together and the solvent was removed to yield a residue that was dried overnight under vacuum. Yields, based on the amount of DMTr-Cl used, ranged from 60 to 80 %. Products are characterized by standard methods, such as NMR spectroscopy and/or elemental analysis.

In this manner, the following tritylated diol linkers were obtained from the reagents noted below:

(A): DMTr-O-$(CH_2)_9$-OH, yield 76.4% from 1,9-nonanediol [3.6 g (22.5 mmol)]; DMTr-Cl [5.0 g (15 mmol)]; and pyridine [100 ml].

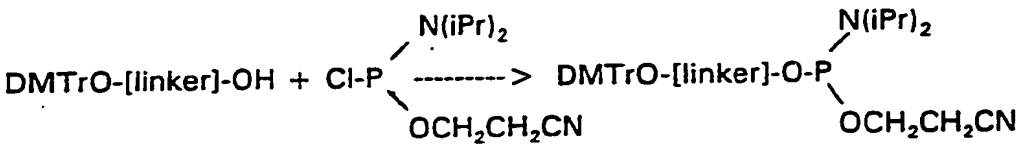
(B): DMTr-O-$(CH_2)_2$-O-$(CH_2)_2$-O$(CH_2)_2$-OH, yield 68.2% from triethylene glycol [3.4 g (22.5 mmol)]; DMTr-Cl [5.0 g (15 mmol)]; and pyridine [100 ml].

(C): DMTr-O-$(CH_2)_3$-OH, yield 67.0% from 1,3-propanediol [1.7 g (22.5 mmol)]; DMTr-Cl [5.0 g (15 mmol)]; and pyridine [100 ml].

(D) DMT-O-$(CH_2CH_2O)_5$-CH_2CH_2-OH, yield 68.4% from hexaethylene glycol [6.35g (22.5 mmol)], DMT-Cl [5 g (15 mmol)], and pyridine (100 ml).

Example 2 - Phosphitylation of tritylated linkers

The tritylated linker prepared as described in Example 1 was next derivatized at the remaining hydroxyl group to incorporate a phosphoramidite group, according to the reaction scheme illustrated below:



(i) The tritylated product obtained from previous preparations (1-5 mmol) was dissolved in dry THF (10-50 ml). Anhydrous diisopropylethylamine (DIPEA) (4-20 mmol, 4 eq.) was injected under a weak flow of argon.

(ii) The phosphitylating reagent 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (2-10 mmol, 2 eq., Aldrich Chemical Co.) was then added with a syringe over a period of 2-5 minutes. A white precipitate was quickly formed.

(iii) The reaction mixture was stirred at room temperature for 1-2 hours and monitored by TLC (EtOAc/CH_2Cl_2/TEA, 45:45:10, v/v).

(iv) When the reaction had gone to completion, the excess phosphitylating reagent was quenched by adding several ice cubes. The mixture was diluted with ethyl acetate (50-250 ml) and triethylamine (1-5 ml). The solution was then transferred to a separatory funnel and extracted twice with 10% aqueous sodium carbonate and twice with saturated aqueous sodium chloride.

(v) The organic phase was dried over anhydrous sodium sulphate, filtered, and then evaporated to dryness under reduced pressure.

(vi) The residue was purified by flash chromatography on silica gel using petroleum ether /EtOAc/TEA (20:10:1, v/v) as eluant.

(vii) Fractions containing pure product were combined, evaporated and then dried overnight under high vaccum to remove traces of triethylamine. The product was stored at -20 °C. Yield of the isolated product varied from 65 to 80 %. Product is characterized by standard methods, such as ^{1}H-NMR ^{31}P-NMR, and elemental analysis.

In this manner, and using the tritylated products of example 1 as starting material, there were prepared the following linkers suitable for coupling between nucleotides via the phosphoramidite approach:

Linker A: DMT-O-$(CH_2)_9$-O-phosphoramidite

TLC (silica gel, petroleum ether/EtOAc/TEA, 50:10:1, v/v/v): R_f 0.84, ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.14-1.62 [26H, m, $C\underline{H}_2$, $CH(C\underline{H}_3)_2$]; 2.63 (t, 2H, J = 6.5 Hz, $C\underline{H}_2CN$); 3.02 (t, 2H, J = 6.5 Hz, $DMTOC\underline{H}_2$); 3.54-3.88 [2m with one s centred at 3.78, 12H, $OC\underline{H}_3$, $C\underline{H}_2OP$, $POC\underline{H}_2CH_2CN$, $NC\underline{H}(CH_3)_2$]; 6.79-6.84 (m, 4H, arom. H ortho of OCH_3); 7.17-7.45 (m, 9H, arom. H). ^{31}P-NMR ($CDCl_3$, 121 MHz): 122.4 ppm.

Linker B: DMT-O-$(CH_2CH_2O)_2$-CH_2CH_2-O-phosphoramidite

TLC (silica gel, petroleum ether/EtOAc/TEA, 50:10:1, v/v/v): R_f 0.48, ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.13-1.18 [12H, 2d, $CH(C\underline{H}_3)_2$]; 2.51-2.64 (m, 2H, $C\underline{H}_2CN$); 3.23 (t, 2H, J = 5 Hz, $DMTOC\underline{H}_2$); 3.56-3.86 [m, 20H, $OC\underline{H}_3$, $OC\underline{H}_2C\underline{H}_2O$, $C\underline{H}_2OP$, $POC\underline{H}_2CH_2CN$, $NC\underline{H}(CH_3)_2$]; 6.77-6.86 (m, 4H, arom. H ortho of OCH_3); 7.18-7.47 (m, 9H, arom. H). ^{31}P-NMR ($CDCl_3$, 121 MHz): 148.6 ppm.

Linker C: DMT-O-$(CH_2)_3$-O-phosphoramidite

TLC (silica gel, petroleum ether/EtOAc/TEA, 50:10:1, v/v/v): R_f 0.79, ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.00-1.30 [12H, 2d, $CH(C\underline{H}_3)_2$]; 1.89-1.97 (m, 2H, $CH_2C\underline{H}_2CH_2$); 2.44-2.51 (m, 2H, $C\underline{H}_2CN$); 3.14-3.19 (m, 2H, $DMTOC\underline{H}_2$); 3.50-3.88 [m, 12H, $OC\underline{H}_3$, $C\underline{H}_2OP$, $POC\underline{H}_2CH_2CN$, $NC\underline{H}(CH_3)_2$]; 6.74-6.84 (m, 4H, arom. H ortho of OCH_3); 7.14-7.47 (m, 9H, arom. H). ^{31}P-NMR ($CDCl_3$, 121 MHz): 147.3 ppm.

Linker D: DMT-O-$(CH_2CH_2O)_5$-CH_2CH_2-O-phosphoramidite

Linker D: DMT-O-$(CH_2CH_2O)_5$-CH_2CH_2-O-phosphoramidite

TLC (silica gel, petroleum ether/EtOAc/TEA, 50:10:1, v/v/v): R_f 0.12, ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.15-1.21 [12H, 2d, $CH(C\underline{H}_3)_2$]; 2.57-2.66 (m, 2H, $C\underline{H}_2CN$); 3.23 (t, 2H, J = 5 Hz, $DMTOC\underline{H}_2$); 3.56-3.91 [m, 32H, $OC\underline{H}_3$, $OC\underline{H}_2C\underline{H}_2O$, $C\underline{H}_2OP$, $POC\underline{H}_2CH_2CN$, $NC\underline{H}(CH_3)_2$]; 6.76-6.85 (m, 4H, arom. H ortho of OCH_3); 7.16-7.48 (m, 9H, arom. H). ^{31}P-NMR ($CDCl_3$, 121 MHz): 148.6 ppm.

Example 3 - General procedure for linear polynucleotide conjugate synthesis

Controlled pore glass (CPG) was used as the solid support matrix for both DNA & RNA synthesis. Polydeoxyribonucleotides (DNA) were prepared by the CE-phosphoramidite method on an Applied Biosystems 391 EP synthesizer (0.15 micromole scale). . Cleavage and deprotection were effected by standard ammonia treatment. Oligoribonucleotides (RNA) were prepared according to the method of Usman et al, 1987, J. Am. Chem. Soc., 109, 7845-7854, employing 5'-dimethoxytrityl-2'-t-butyldimethoxysilyl ribonucleoside-3'-CE- phosphoramidites (Peninsula Labs, CA or ChemGenes Corp., MA). Syntheses were carried out on an Applied Biosystems 380B synthesizer using a modified 0.2 micromole cycle. Cleavage from the support, base & phosphate deprotection, and removal of the 2'-TBDMS groups were performed by established procedures (Scaringe et al, 1990, Nucl. Acids Res., 18, 5433-5441). The crude oligonucleotide in TBAF solution was desalted on a C_{18} Sep-Pak cartridge prior to purification.

The linker phosphoramidite (dissolved in dry acetonitrile, 0.2-0.3 M) was coupled to the support-bound polynucleotide at the desired location, using the synthesis cycle conventional for standard nucleoside phosphoramidites.

In one synthesis cycle, the DMTr protecting groups were removed from the extended oligomer with 2.5 % dicholoroacetic acid / dichloromethane. After several washes (acetonitrile is the only solvent used for all washes), cyanoethyl protected nucleoside phosphoramidites (0.12 M in dry acetonitrile) were coupled to the support in the presence of 0.5 M tetrazole. The coupling time for DNA oligomers was 15 sec (ABI 391EP) and 2 x 6 minutes for RNA oligomers (ABI 380B). Double couplings were used for RNA synthesis since these phosphoramidites are much less reactive than their DNA homologs. This is followed by capping of the unreacted hydroxy groups (Ac_2O/DMAP), and oxidation of the phosphite triesters to the phosphates (I_2/H_2O). The cycles were repeated until the desired polynucleotide conjugate was obtained. The conjugate was then cleaved from the CPG support by treatment with concentrated ammonia for one hour at room temperature. Deprotection of DNA conjugates and of RNA conjugates was achieved by incubation in ammonia at 55°C for 6-16 hours. For RNA conjugates specifically, deprotection was performed with ammonia in ethanol (3:1), and a final treatment involved incubation in 1M TBAF at room temperature. The average coupling yield, as assayed by trityl measurement, was 97-99 % for DNA oligos, and 95 - 97 % for RNA oligos.

A summary of the protocols used in RNA conjugate synthesis is provided in Table 1 below, for convenience:

TABLE I: Synthetic cycle for the preparation of linker-derivatized TAR oligoribonucleotides

STEP	REAGENT OR SOLVENT	PURPOSE	TIME (sec)
1	Dichloroacetic acid in CH_2Cl_2 (2.5:97.5; v/v)	Detritylation	5 x 20

2	Anhydrous CH_3CN	Wash	90
3	Activated phosphoramidites in anhydrous CH_3CN *	Coupling	2 x 360
4	Anhydrous CH_3CN	Wash	20
5	HPLC grade CH_2Cl_2	Wash	20
6	Anhydrous CH_3CN	Wash	20
7	DMAP/THF (6.5 g: 94 ml) Ac_2O/Lutidine/THF (1:1:8; v/v/v)	Capping	60
8	0.1 M I_2 in THF/Lutidine/H_2O (160:40:4; v/v/v)	Oxidation	60
9	Anhydrous CH_3CN	Wash	3 x 20

* The coupling reactions were carried out by pre-mixing 0.5 M tetrazole with 0.15 - 0.30 M standard or modified phosphoramidites in anhydrous CH_3CN.

The crude deprotected polynucleotide conjugates were purified by standard electrophoresis methods (Atkinson & Smith, in (1984) "Oligonucleotide Synthesis: A Practical Approach" (Gait, M.J.; ed.) IRL Press, Oxford/Washington, D.C.)using 15-20 % polyacrylamide / 7M urea gels. The bands were visualized by UV shadowing and the product was cut out and eluted from the gel. The eluted conjugate was finally desalted on a C_{18} Sep-Pak and quantitated by OD_{260}.

Each oligonucleotide linker conjugate was checked for homogeneity and "sized" by 5'-^{32}P-end labeling / analytical PAGE against the crude material and oligonucletide markers. These RNA oligomers were further characterized by enzymatic RNA sequencing [Donis-Keller, H. (1980) Nucleic Acids Res., *8*, 3133-3142] or base-composition analysis [Seela, F. & Kaiser, K. (1987) Nucleic Acids Res., *15*, 3113-3129].

Example 4 - Polynucleotide conjugate synthesis

To evaluate the effect of the chemical linker on the stability and ligand binding properties of a naturally occurring duplex structure, there was first employed a model system comprising DNA strands capable of duplexing to form an EcoRI recognition/cleavage site. As shown below in structure 1, unlinked oligomers constituting the EcoRI site were examined for comparison.

5' - GGAATTCC - 3'
3' - CCTTAAGG - 5' 1

5' - GGAATTCC - linker B - GGAATTCC - 3' 2

5' - GGAATTCC - linker C - GGAATTCC - 3' 3

For comparison, there was prepared linear polynucleotide conjugate 2 which contains triethylene glycol-derived linker B, having the structure -O-$(CH_2)_2$-O-$(CH_2)_2$-O-$(CH_2)_2$-O; and linear polynucleotide conjugate 3 which contains propanediol-derived linker C, having the structure -O-$(CH_2)_3$-O-. If the length and nature of linker B has been selected appropriately, polynucleotide conjugate 2 should adopt a duplexed structure that is digested more rapidly by EcoRI than the unlinked control molecule 1. The linker C in conjugate 3 is expected to be too short to permit functional annealling of the strands, which should translate into slower EcoRI digestion relative to conjugate 2. The conjugates were

prepared using the protocols described above in example 3 and then radiolabelled, and the EcoRI digestion reaction was monitored, in the following manner:

Polynucleotide labelling: oligonucleotides (5 pmol) were dissolved in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM KCl and 5 mM dithiothreitol (DTT) and incubated with 9 pmol of γ-^{32}P-labelled ATP and 10 units of T4 polynucleotide kinase (New England Biolabs) at 37°C for 1-2 h. The reaction was terminated by heating the mixture to 80°C for 10 min, and then was slowly cooled to room temperature. The solution was desalted by passage through a Bio-spin column (BIO-RAD, Bio-spin 6 for the unlinked control, and Bio-spin 30 for the polynucleotide conjugates). An alternative method for purification of labelled polynucleotide involved one-time extraction with an equal volume of phenol solution and precipitation using two volumes of ethanol/acetate (1:1; v/v) at -20°C overnight, with collection and drying under high speed vacuum.

EcoRI digestion: 1 pmol of the selected, ^{32}P-labelled substrate was incubated with 20 units of EcoRI (Pharmacia) in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM βME and 100μg BSA/ml (total volume: 20μl). The reactions were carried out at room temperature, and 2μl of sample was removed at different time intervals. The samples were analyzed on a 20% denaturing polyacrylamide gel.

Under these conditions, 60% of the unlinked control was digested after 24 h whereas conjugate 2 was completely digested within 11 hours. The increase in digestion rate is approximately 6-8 fold faster with conjugate 2, owing to enhanced stability of the duplexed structure. As expected, digestion of conjugate 3 was very slow (only about 5% of starting material was digested after 24 h incubation). These results indicate clearly that a linker of appropriate length can significantly

enhance the stability of and retain the function of duplexed structures, including those having protein binding affinity.

Example 5 - Production of RNA polynucleotide conjugates

The RNA structure known as Tar consists of 59 bases in most HIV-1 isolates, arranged in a stem-loop structure with two or three bulges in the stem. Previous studies have shown however that the full length Tar structure can be reduced significantly in size to a 27-mer (Fig.4) while retaining full tat-binding activity (Sumner-Smith et al, J. Virol., 1991, 65:5196.

Various linear polynucleotide conjugates, representing analogues of a 27-mer truncated version of Tar (Figure 4) were synthesized and evaluated. All were prepared using the synthesis procedures previously described hereinabove. As Figure 4 illustrates, the linear polynucleotide conjugates tested comprised two classes; one class in which the 6-mer loop in the Tar analogue (4) was replaced by each of four different linkers (conjugates 5A, 5B, 5C and 5D) and another class in which the 6-mer loop was replaced by two coupled linkers (5BB and 5CC). The stability and tat binding properties of these oligonucleotides were determined and compared, and the results are shown in Table 2 below.

Melting temperature (Tm) measurements were carried out in 100 mM NaCl/10 mM sodium phosphate buffer (pH 7.0). Samples were heated from 25 to 85°C in 1°C increments using a HP 8459 UV/VIS spectrophotometer and a HP 89100A temperature controller. The concentration of nucleic acid was 2.5-3.0 μM, and absorbance was monitored at 260 nm. T_m values were determined by a first-derivative plot of absorbance *vs* temperature. Each experiment was performed in duplicate and the average reported as the thermal denaturation temperature.

Ligand binding of the oligonucleotides was assessed by gel electrophoresis and RNA mobility shift assay. Linker-derivatized oligoribonucleotides (*5A-5CC*) and the control sequences (*4, 6* and *7*, Fig.4) were 5'-^{32}P-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled oligomers were then purified by phenol/chloroform extraction/EtOH precipitation or spin-column filtration (Bio-Rad, Bio-Spin 30). Prior to binding assays, the RNAs were dissolved in 20 mM Tris-HCl (pH 7.5)/ 100 mM NaCl, heated to 85°C for 3 min, then slow-cooled to room temperature. Binding assays were carried out in 20 μl reaction mixtures containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5 U/ml RNAsin (Promega), 0.09 μg/ml BSA, 5% (v/v) glycerol, 0.1 nM ^{32}P-labeled RNA (2000-5000 cpm) and either peptide derived from the HIV-1 Tat protein RKKRRQRRRPPQGS (amino acids 49-62 of HIV LAI isolate) (Weeks et al., Science, 1990, 249:1281; Delling *et al.*, Proc. Natl. Acad. Sci., 1991, 88:6234) (American Peptide Co., Santa Clara, CA) or full-length Tat protein (American Bio-Technologies, Inc.) at a concentration of 0.5 pM to 1000 nM (Roy *et al.*,Genes Dev., 1990, 4:1365). The reactions were incubated at 23°C for 25 min, chilled on ice for 5 min, then loaded on 5% native polyacrylamide gels (acrylamide:bis-acrylamide = 30:0.8, w/w) containing 5% glycerol. The gels were pre-run for 15 min prior to loading, then run for 2.5 h at a constant current of 30 mA at 4°C in 0.5X TBE buffer. The gels were dried onto DEAE paper (Whatman DE81) and exposed to Kodak X-Omat X-ray film with an intensifying screen overnight at -70°C. Competition binding experiments were carried out as described above except that the concentration of Tat protein was kept constant at 100 nM and unlabeled competitor RNA was added in a concentration range of 0.9 nM to 5000 nM.

TABLE II: Thermodynamic and binding properties of TAR analogues

OLIGOMER	SUBSTITUTION	T_m (°C)	(K_d) BINDING(%)	
4	6-nt loop (wt sequence)	60	+ (0.41)	45.9
5A	linker A / loop	61	+ (0.71)	40.1
5B	linker B / loop	58	+ (0.95)	42.6
5C	linker C / loop	56	-	
5D	linker D / loop	63	+ (0.66)	56.0
5BB	2 X linker B / loop	59	+ (1.13)	38.3
5CC	2 X linker C / loop	56	+ (0.43)	17.8
6 + 7	without connection	32	-	

K_d values are expressed in nanomolar concentrations

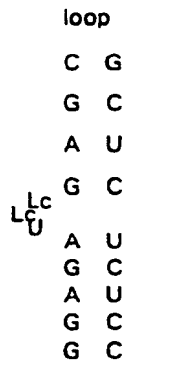
+ strong binding

- no binding

Binding capacity indicates the % of active RNA molecules capable of binding to peptide upon saturation

The thermal denaturation experiments indicated that every linker-derivatized TAR analogue had some secondary structure. With the exception of structure 5C which incorporates a linker expected to be too short to allow proper duplex formation, binding assays revealed tat-binding function in the conjugated duplexes versus the unlinked controls. Similar binding was also confirmed in experiments using the full length tat protein.

Further evaluation of linker incorporation has indicated that relatively short linkers can be used to advantage, to replace nucleotides resident in the polynucleotide strands, e.g. to replace nucleotides in the bulge of TAR. In particular, a Tar conjugate was produced in which the bulge 5'-U-C-U-3' was replaced by the structure 5'-U-L_c-L_c-3', to yield structure 8 (Kd = 0.51nM, Tm = 60°C)) shown below:



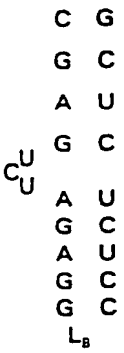
8

where LcLc is -O-$(CH_2)_3$-O-PO_2-O-$(CH_2)_3$-O-.

Tat-binding analysis of the resulting structure has shown that replacement of nucleotides within the bulge preserved the tat-binding structure of TAR. Thus, in certain instances, linkers equivalent in length to C_3 can be used, particularly within the so-called bulge structures which form at non-annealed sites of duplex structures.

Moreover, studies with a short un-linked duplex (oligomer 6 + 7) of same length have shown that this duplex has a significant lower T_m (32°C) when compared to its linked counterparts (56 - 63°C), and it also failed to form any effective complexes with Tat-derived peptide, probably due to its thermal instability. This provides strong evidence that synthetic linkers add substantial stability to the un-linked duplex structures to a such degree that their normal biological functions, such as binding to proteins, can be maintained.

In another experiment, there was successfully generated a particular Tar analog where the linker was incorporated at the bottom of the duplex (oligomer 9, below).



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Both T_m measurements (T_m = 61°C) and binding assays (K_d = 2.20nM) indicated that this analog also retains the physical and binding properties of the wild-type Tar structure.

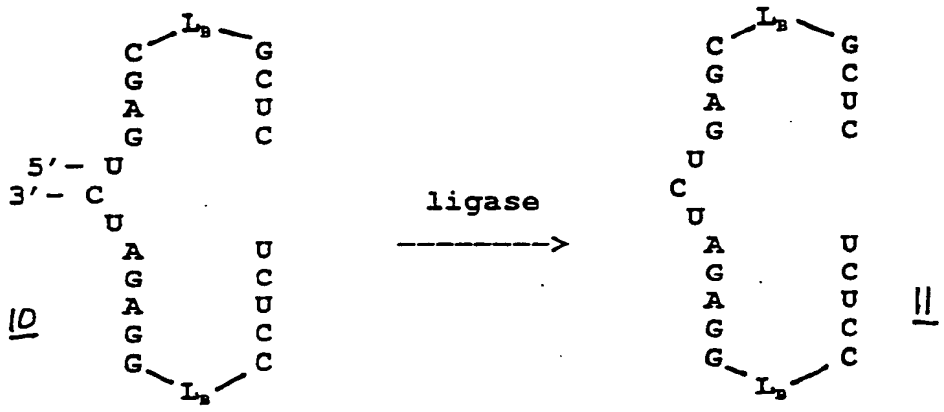
Example 6 - Binding assays with full-length Tat and competition experiments

To evaluate possible differences in binding affinity for the short Tat-peptide and full length native Tat protein, the binding affinities of the Tar conjugates for full-length Tat protein (86 amino acids) were assessed using the mobility shift assay. By this method, The K_d value for the full-length Tat (1.17 nM) was slightly higher than that for the Tat-derived peptide (0.71 nM). When Tar conjugate 5B was added to a pre-formed complex between the 27mer fragment of the wild-type Tar stem-loop (oligomer 4) and full-length Tat protein, strong competition with the TAR sequence was observed. The complex was totally competed away when the ratio between the Tar conjugate and the Tat protein was 1:1.

Example 7 - Synthesis of cyclic polynucleotide conjugates

To synthesize cyclic polynucleotide conjugates, there was applied the general approach of (a) synthesizing the corresponding linear

polynucleotide conjugate in the manner described previously herein, and then (b) cyclizing the linear polynulceotide conjugate either via enzymatic ligation (DNA or RNA ligase) or by chemical closure. In particular, the enzymatic ligation approach has been applied to convert linear conjugate 10, to the cyclic TAR conjugate 11, as shown below:



To prepare the cyclic analogue, the linear conjugate 10 was first radiolabelled with gamma ^{32}P-ATP as described previously herein. The heated T4 polynucleotide mixture was then cooled slowly to room temperature, and 1μl (10 units) of T4 RNA ligase were then mixed with 10μl of radiolabelled conjugate, 2μl of ATP (10mM) and 7 ul of 1X ligase buffer consisting of 66mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. The ligation reaction was pursued for four hours at room temperature.

The ligated product was then purified on a 20% denaturing polyacrylamide gel. The band corresponding to the cyclic conjugate (evident from its faster migration relative to linear conjugates) was cut out and extracted from the gel with 0.3 M NaOAc at room temperature overnight. The sodium acetate solution containing the cyclic conjugate was then washed with an equal volume of phenol solution in order to eliminate any proteinaceous contamination. After this step, two volumes

of ethanol/acetone (1:1, v/v) solution were added to the aqueous phase, and the mixture was stored at -20°C overnight. The cyclic conjugate 11 was ultimately collected and was dried under high speed vacuum.

Example 8 - Ligation site optimization for generating cyclic conjugates

To cyclize the polynucleotide conjugates as efficiently as possible a number of potential ligation sites (a-e) were examined using structure 12 (Fig. 5). To prepare this cyclic polynucleotide conjugate, the linear conjugates (one for each ligation site chosen) were synthesized and radiolabelled with gamma ^{32}P-ATP as described previously herein. 10 μl of each radiolabelled conjugate was added to 2ul of ATP (10mM), 2μl of DMSO(100%), 2μl of 10X ligase buffer consisting of 500mM Tris-HCl (pH 7.8). 100mM $MgCl_2$ 100mM ß-mercaptoethanol, 10mM ATP, and 1μl (10 UNITS) of RNA Ligase. The ligation reaction was pursued for 4 hours at 37°C. The ligated products (2μl of each) were examined by separation on 20% denaturing polyacrylamide and compared directly to an equivalent amount of unligated linear radiolabelled polynucleotide conjugate on the same gel.

From these results, it was determined that ligation site c (between the A and G residue) on the front strand immediately beneath the -UCU- bulge gave the best conversion of linear to cyclic conjugate.

Example 9- Binding properties of cyclic polynucleotide conjugates

Using the best ligation site identified from the previous example, there was successfully generated a series of Tar conjugates; two of them are illustrated in Figure 6. Both of these constructs (14 & 15) are 21-mers and differ only in the chemical linker used to replace the nucleotide loops at the top and bottom of the duplex. Oligomer 14 contains Linker A and oligomer 15 contains linker D. All three cyclic polynucleotide conjugates were subjected to the binding assay as described previously.

It was found that the 31-mer (oligomer 13) as well as the linker D cyclic conjugate (oligomer 15) bind efficiently to both peptide and the full-length Tat protein, although, for reasons that are not clear, no binding was seen with oligomer 14. It is possible that while the length of the chemical linker used does not appear to be significant in the linear series, it may be significant for proper functioning of cyclic polynucleotide conjugates that bind to Tat protein. This suggests that synthetic duplex-stabilizing linkers should have some flexibility in order to allow the mini-duplexes to adopt possible conformational changes upon protein recognitions.

Example 10 - *In Vitro* Stability and cell uptake of polynucleotide conjugates

A number of different polynucleotides were used in a comparative analysis of the relative stability of linear versus cyclic conjugates. For these studies the following conjugates were used (Fig. 7). To evaluate conjugate stability further there was also generated a 21-mer RNA oligomer (5'-CUUCGCAGUAUGUUAGCCGGU-3') which has the same base composition as the cyclic oligomers 14 & 15, but should remain in single-stranded open-circle form due to the non-complementarity between the bases (Figure 7, oligomer 16). Each of the polynucleotide conjugates used was synthesized, radiolabelled, and ligated as previously described herein. After radiolabelling and/or ligation, the polynucleotides were purified on 20% denaturing polyacrylamide gels as previously described. For each of the various conditions, the same amount of radiolabelled gel-purified polynucleotide was used (300,000 CPM). The conditions used for each of the reactions are described below.

Exounuclease III: 300,000 CPM of gel-purified polynucleotide was incubated in the presence of 20 units of Exonuclease III (1μl) and 1μl of 10X buffer which consisted of 500mM Tris-HCl pH 8.0, 50mM $MgCl_2$,

100mM ß-mercaptoethanol. Enzymatic treatment was pursued for 6 h at 37°C and a sample was removed for analysis at this time.

Mung Bean Nuclease: 300,000 CPM of gel-purified polynucleotide was incubated in the presence of 5 units of Mung Bean Nuclease (1μl) and 1μl of 10X buffer which consisted of 500mM sodium acetate pH 5,0, 300mM NaCl, 1mM $ZnSO_4$. Enzymatic treatment was pursued for 6 h at 37°C and a sample was removed for analysis at this time.

Calf Intestinal Alkaline Phosphatase: 300,000 of CPM gel-purified polynucleotide was incubated in the presence of 5 units of calf intestinal alkaline phoshatase and 1μl of 10X buffer which consisted of 500mM Tris-HCl pH 8.5, and 1mM EDTA. Enzymatic treatment was pursued for 20 h at 37°C and a sample was removed at this time.

Cell Extract and Nuclear Extracts: Cell and nuclear extracts were prepared essentially by the method of Dignam et. al., 1983, Nucl. Acids. Res., 11:1475. The amount of protein in each extract was determined using Bovine Serum Albumin as a standard. 300,000 CPM of gel-purified polynucleotide was incubated in the presence of 8ug cell extract protein, or 6μg nuclear extract protein at 37°C. Equivalent samples from both cell and nuclear extract digestions were removed at various times (8 and 24 h).

Samples from all treatments were applied to 20% denaturing polyacrylamide gels and exposed to Kodak X-Omat AR film. The band of interest was excised from the gel and the amount of radioactivity was determined. The relative stability of each treatment was determined by comparing the amount of radioactivity of each sample to the amount of radioactivity of a control sample which was not treated with the same enzyme. Results of these stability studies are presented below:

Table III: Stability studies of TAR conjugates

TREATMENT*	TIME (h)	#4	#5A	#16	#13	#14	#15
EXONUCLEASE III	6	8.0%	19%	14%	46%	79%	84%
MUNG BEAN	6	3.0%	27%	8.5%	35%	49%	54%
CIAP	24	+	+	-	-	-	-
CELL EXTRACT	8	4.8%	1.7%	2.4%	72%	73%	86%
	24	0.2%	0.3%	0.5%	34%	55%	37%
NUC. EXTRACT	8	1.0%	1.5%	33%	83%	88%	84%
	24	0.1%	0.3%	0.5%	58%	31%	32%

All treatments were carried out at 37 °C. Cellular and nuclear extracts were obtained from HEP-2 cells (liver cells).

\+ Sensitive to dephosphorylation by CIAP treatment.

\- Not sensitive to dephosphorylation by CIAP treatment.

% # of full length molecules remaining

These results demonstrate that Tar conjugate 5A has a similar stability as the wild-type sequence (oligomer 4) in cellular and nuclear extracts although the conjugate appears far more stable against single strand-specific nucleases such as mung bean nucleases. The duplex-forming cyclic linker molecules (oligomer 14 & 15) are much more stable than both the linear conjugates and the single-stranded cyclic control (oligomer 16).

Cell uptake studies

For these studies, 5 pmoles of the Linker A and Linker D conjugates (oligomer 14 & 15 in Figure 7) were radiolabelled, ligated, and gel-purified as described previously. For each of these polynucleotides, equivalent amounts of radioactivity were incubated in the presence of Hut-78 (Human T-cells) and at various times, samples were removed and the radioactivity in various fractions was determined. The percentage of uptake was plotted over time and results are shown in Figure 8 for oligomer 14 and in Figure 9 for oligomer 15. As the Figures illustrate, approximately 20% of the polynucleotide is cell-associated after 24 hours of incubation.

WE CLAIM:

1. A duplex-forming, polynucleotide conjugate, comprising a first polynucleotide strand having an end, a second polynucleotide strand which is capable of annealing with the first polynucleotide strand and having an end, and a chemical linker which is coupled covalently between said ends to form a bridge permitting the first and second polynucleotides to form a ligand binding, duplexed structure.

2. A duplex-forming, polynucleotide conjugate according to claim 1, selected from among the group consisting of:

(i) a linear polynucleotide conjugate of the formula;

X - L - Y (I)

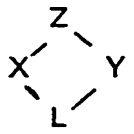
wherein:

X is a polynucleotide having a 3'terminus;

Y is a polynucleotide capable of annealing with X, and having a 5'terminus; and

L is a chemical linker coupled covalently between the 3'terminus of X and the 5'terminus of Y to form a bridge permitting X and Y to form a ligand-binding duplexed structure;

(ii) a cyclic polynucleotide conjugate of the formula:



(IIa)

wherein:

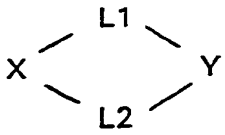
X is a polynucleotide having a 5'terminus and a 3'terminus;

Y is a polynucleotide capable of annealing with X and having a 3'terminus and a 5'terminus;

Z is a polynucleotide coupled covalently between the 5'terminus of X and the 3'terminus of Y; and

L is a chemical linker coupled covalently between the 3'terminus of X and the 5'terminus of Y, to form a bridge permitting X and Y to form a ligand-binding duplexed structure; and

(iii) a cyclic polynucleotide conjugate of the formula:



(IIb)

wherein:

X and Y are as defined above; and

L1 and L2 are independently selected chemical linkers coupled, respectively, between the 3'terminus of X and the 5'terminus of Y and the 5'terminus of X and the 3'terminus of Y, to form chemical bridges permitting X and Y to form a ligand-binding duplexed structure.

3. A polynucleotide conjugate according to claim 2, which in duplexed form binds a protein ligand.

4. A duplex-forming polynucleotide conjugate according to claim 3, which in duplexed form binds a protein that regulates gene expression.

5. A duplex-forming polynucleotide conjugate according to claim 4, which in duplexed form binds a protein that regulates viral gene expression.

6. A duplex-forming polynucleotide conjugate according to claim 5, which in duplexed form binds to the HIV tat protein.

7. A duplex-forming polynucleotide conjugate according to claim 3, which in duplexed form presents an immunogenic epitope.

8. A duplex-forming polynucleotide conjugate according to any one of claims 3-7, wherein said conjugate is a linear polynucleotide conjugate of formula (I).

9. A duplex-forming polynucleotide conjugate according to claim 8, wherein said chemical linker is equivalent in length to an alkane having from 7 to 20 carbon atoms.

10. A duplex-forming polynucleotide conjugate according to claim 9, wherein X and Y comprise a region of mismatched nucleotide sequence forming a bulge structure when in duplexed form.

11. A duplex-forming polynucleotide conjugate according to claim 10, which binds with the HIV tat protein.

12. A duplex-forming polynucleotide conjugate according to any one of claims 3-7, wherein said conjugate is a cyclic polynucleotide conjugate of formula (IIb).

13. A duplex-forming polynucleotide conjugate according to claim 12, wherein X and Y are polynucleotides having a precisely complementary nucleic acid sequence.

14. A duplex-forming polynucleotide conjugate according to claim 13, wherein said chemical linker is equivalent in length to an alkane having from 7 to 20 carbon atoms.

15. A duplex-forming polynucleotide conjugate according to claim 14, wherein X and Y comprise a region of mismatched nucleotide sequence forming a bulge structure when in duplexed form.

16. A duplex-forming polynucleotide conjugate according to claim 15, which binds to the HIV tat protein.

17. A duplex-forming polynucleotide conjugate according to any one of claims 3-7, wherein said conjugate is a cyclic polynucleotide conjugate of formula (IIa).

18. A duplex-forming polynucleotide conjugate according to claim 17, which binds the HIV tat protein.

19. A duplex-forming polynucleotide conjugate according to any preceding claim, wherein X and Y are polynucleotide monophosphates.

20. A duplex-forming polynucleotide conjugate according to claim 19, wherein X and Y are both polydeoxyribonucleotides.

21. A duplex-forming polynucleotide conjugate according to claim 19, wherein X and Y are both polyribonucleotides.

22. A process for preparing a cyclic, duplex-forming, polynucleotide conjugate as defined in claim 17, which comprises the steps of synthesizing a linear analogue thereof in which two chemical linkers permitting the conjugate to form a duplexed, ligand-binding structure are incorporated between polynucleotides X and Y, and then cyclizing the resulting linear polynucleotide conjugate by chemical or enzymatic means.

23. A pharmaceutical composition comprising a duplex-forming, polynucleotide conjugate according to any one of claims 3-21, and a pharmaceutically acceptable carrier.

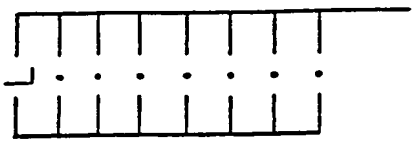


FIG.1(d).

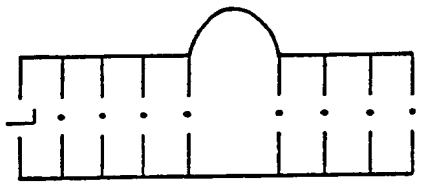


FIG.1(c).

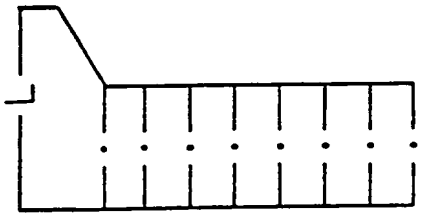


FIG.1(b).

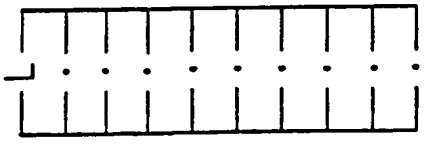


FIG.1(a).

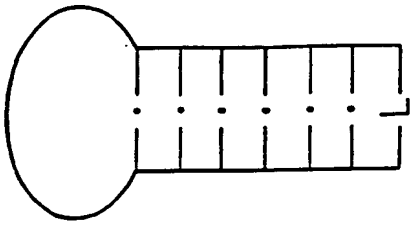


FIG.2(f).

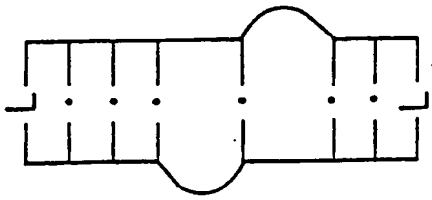


FIG.2(e).

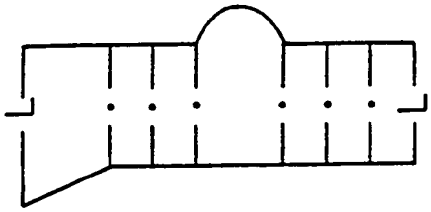


FIG 2(d)

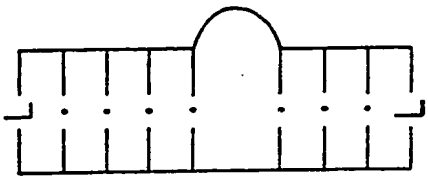


FIG.2(c).

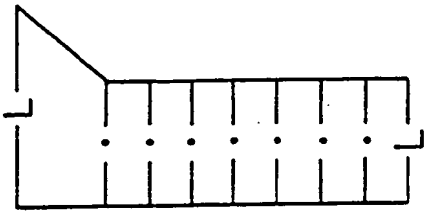


FIG.2(b).

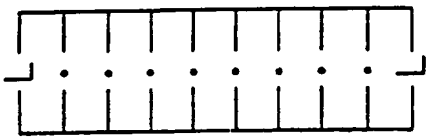


FIG.2(a).

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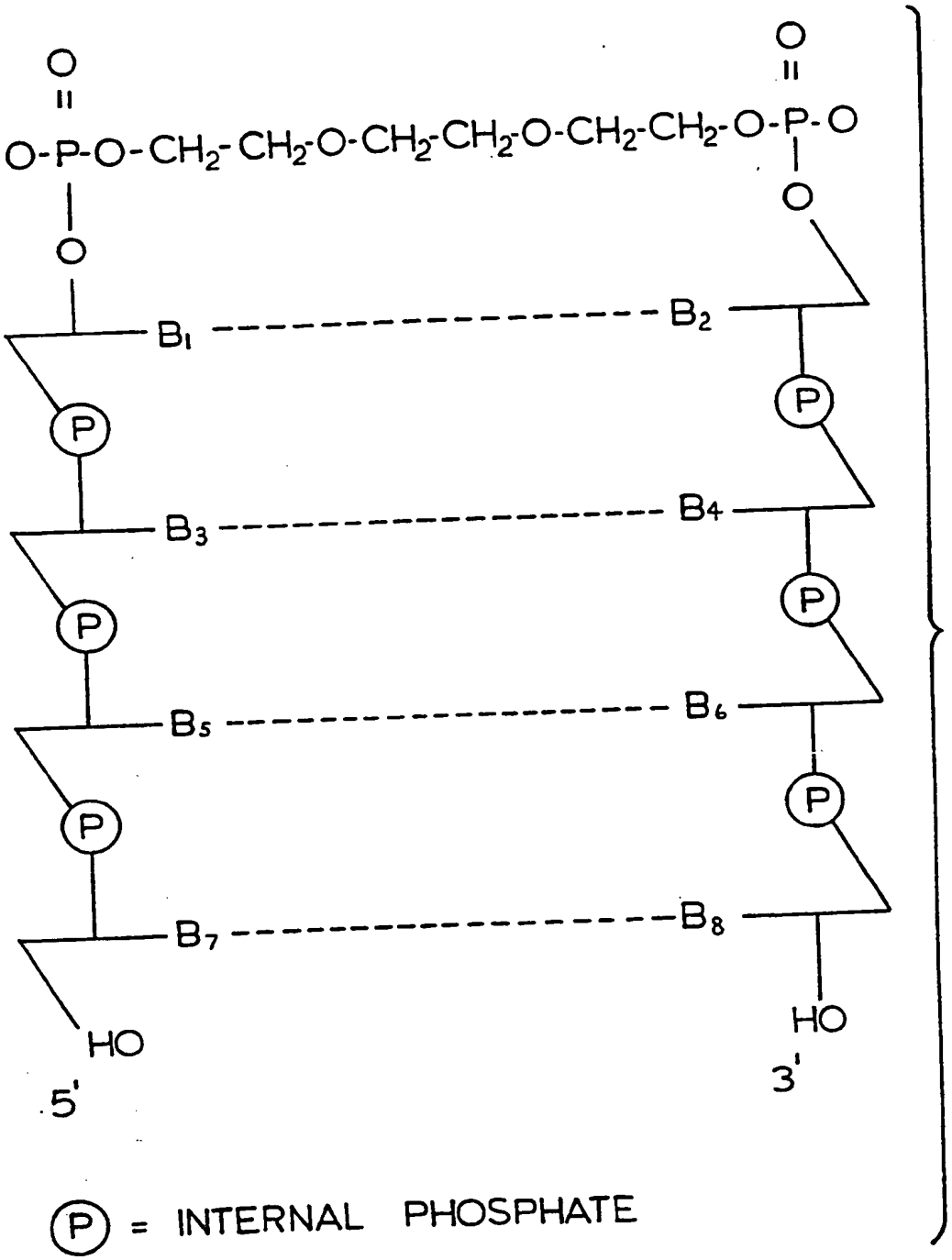
FIG. 3.



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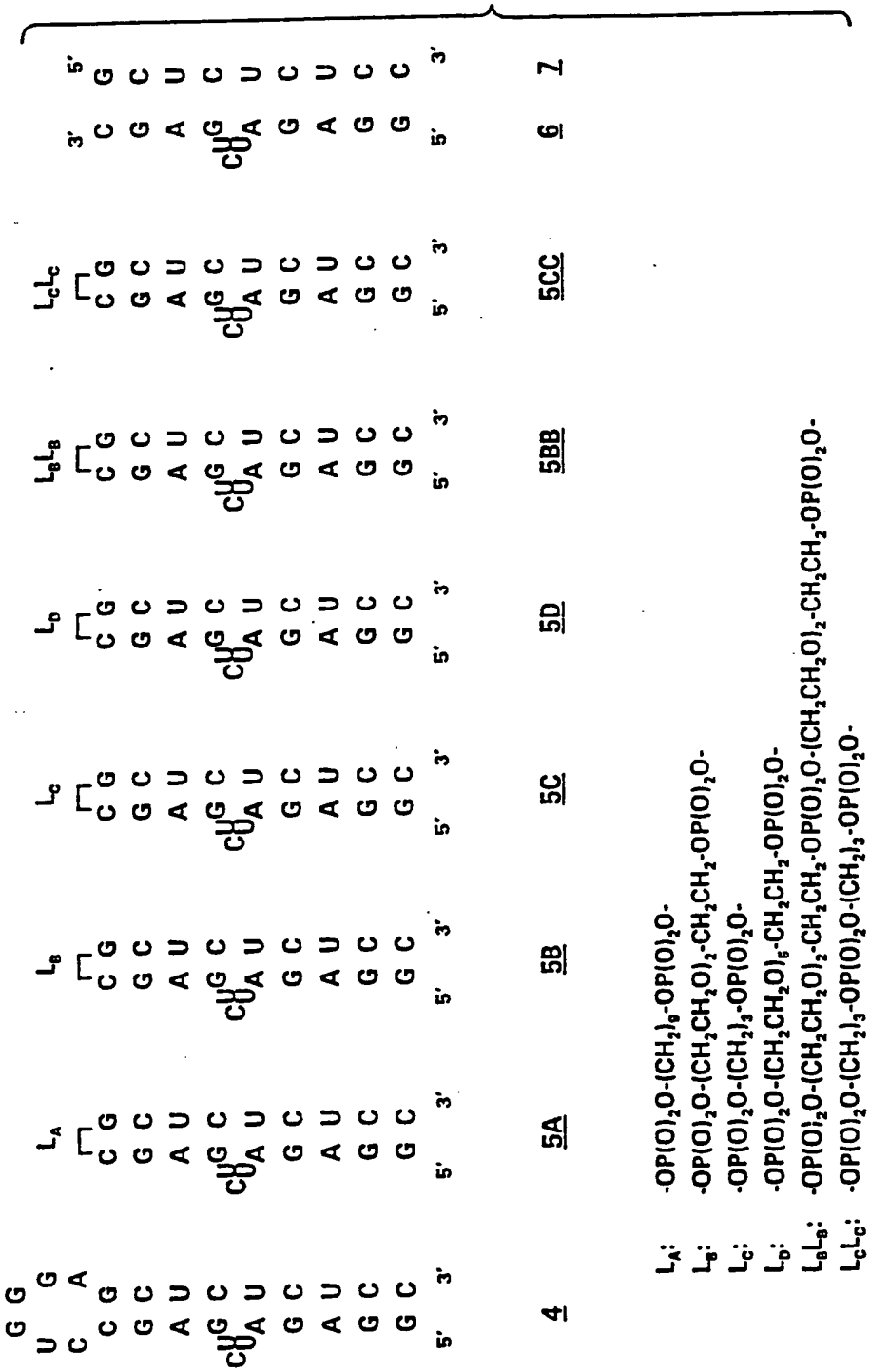

FIG. 4.

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FIG.5.

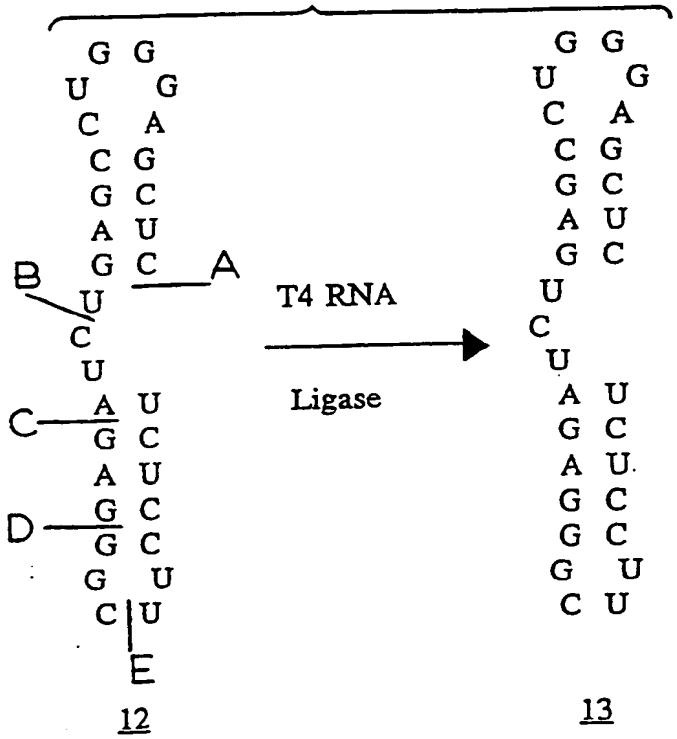

FIG.6.

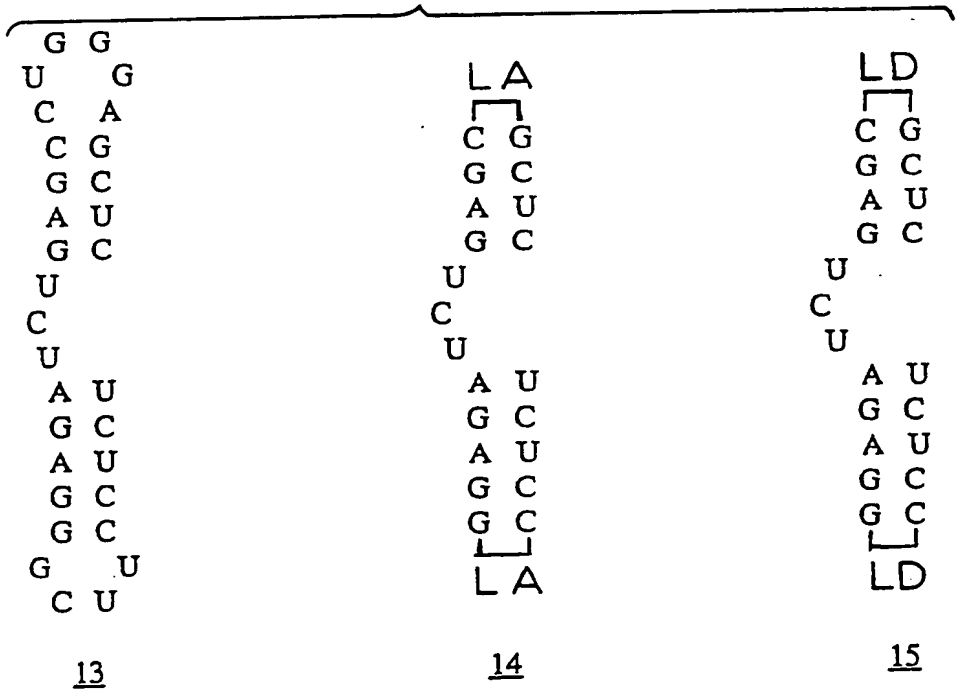

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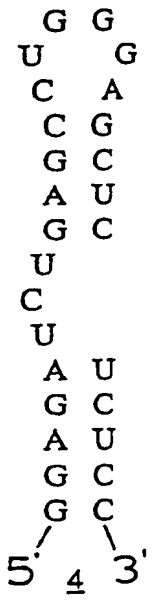
FIG. 7.



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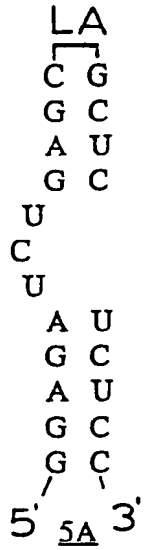
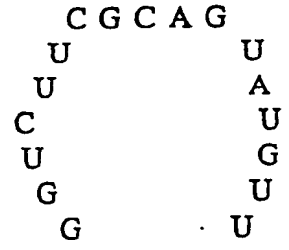


5A



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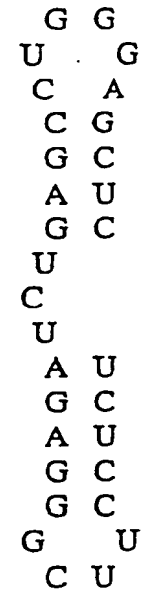

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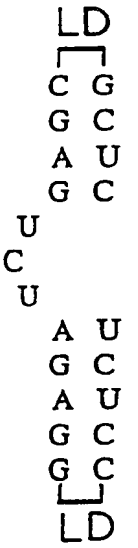

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FIG. 8.

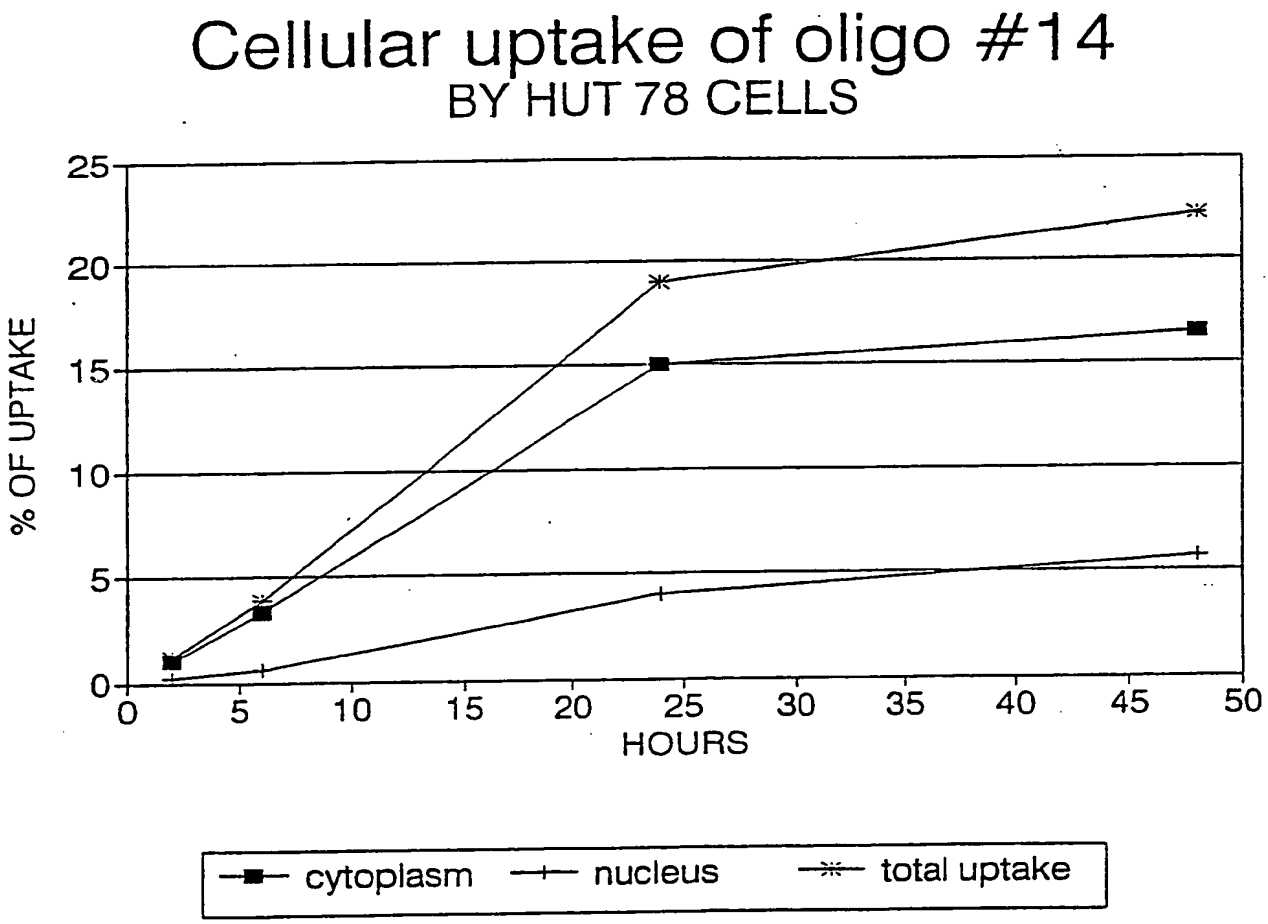

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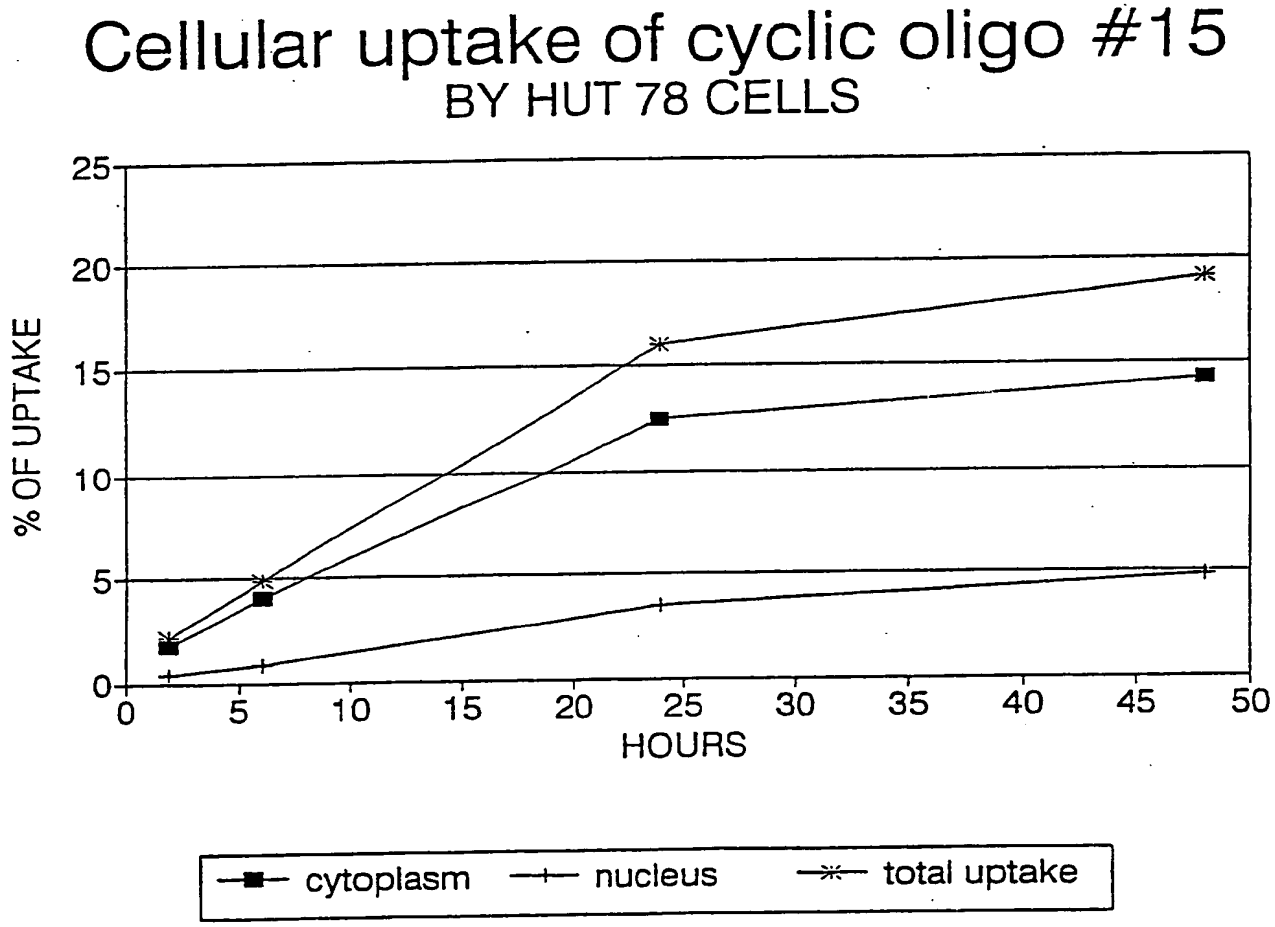
FIG. 9.



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INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 92/00423

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 H 21/04, C 07 H 21/00, C 12 P 19/34, A 61 K 48/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 H; C 12 P; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	Dialog Information Services, file 155, Medline, Dialog Accession No. 07241406, Medline Accession No. 90148406, Cieplak P et al: "Conformations of duplex structures formed by oligodeoxynucleotides covalently linked to the intercalator 2-methoxy-6-chloro-9-aminoacridine", J Biomol Struct Dyn, Oct 1987, 5 (2) p 361-82	1-23
A	Dialog Information Services, file 155, Medline, Dialog Accession No. 05283440, Medline Accession No. 84207440, Lathe R et al: "Linker tailing: unphosphorylated linker oligonucleotides for joining DNA termini", DNA 1984, 3 (2) p 173-82	1-23

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28th December 1992	12. 01. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Carolina Palmcrantz

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	WO, A1, 8500621 (KÖSTER, HUBERT) 14 February 1985, see the whole document --	1-23
A	WO, A2, 8707300 (WORCESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY) 3 December 1987, see especially claims 1-2 --	1-23

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/CA 92/00423

SA 64974

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 02/12/92 The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 8500621	14/02/85	AU-D-	3107084	04/03/85
		DE-A-	3326520	31/01/85
		DE-A-T-	3474566	17/11/88
		EP-A-B-	0149634	31/07/85
		JP-T-	60501838	31/10/85
WO-A2- 8707300	03/12/87	AU-D-	7487787	22/12/87
		EP-A-	0412964	20/02/91
		US-A-	4806463	21/02/89

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

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